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Quantitative DNA Analysis of Low-Level Hepatitis B Viremia in Two Patients With Serologically Negative Chronic Hepatitis B

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Low-level viremia due to hepatitis B virus (HBV) was demonstrated in the sera of two patients diagnosed previously as having non-B, non-C chronic hepatitis. Both patients had a "silent" HBV infection, because they were negative for both hepatitis B surface antigen (HBsAg) and anti-hepatitis B core antibody. The TaqMan chemistry polymerase chain reaction (PCR) amplified the HBV DNA, enabling quantitation of the virus in their sera. Their serum HBV DNA concentrations were low: the amount of each HBV S or X gene amplified showed there were approximately 10^3 copies/ml and HBV DNA was detected occasionally during clinical follow-up. Positive HBsAg staining in liver tissues was demonstrated by an immunoperoxidase technique. Vertical transmission of silent HBV from one patient to her daughter was confirmed. Direct nucleotide sequencing of the amplified HBV X region revealed several mutations, suggesting reduced viral replication. One patient had a T-to-C mutation at the extreme 5′-terminus of the direct repeat 2 region and the other exhibited a coexisting X region with a 155-nucleotide deletion. These findings suggest that HBV replication is suppressed considerably in patients with silent hepatitis B. ***J. Med. Virol. 58: 325–331, 1999.***

KEY WORDS: silent B; HBV mutant; X gene; PCR; quantitation

INTRODUCTION

With the introduction of the polymerase chain reaction (PCR) technique for amplification of hepatitis B virus (HBV) DNA [Kaneko et al., 1990; Liang et al., 1990], HBV infection has been shown to be one of the causative agents in patients diagnosed as having hepatitis of unknown origin and lacking serological markers [Wright et al., 1992; Uchida et al., 1993]. Recent studies have revealed that infection with an HBV mutant was responsible for the pathogenesis of "silent" hepatitis B in patients who were negative for both hepatitis B surface antigen (HBsAg) and anti-hepatitis B core antibody (anti-HBc) [Lai et al., 1989; Kremsdorf et al., 1993; Uchida et al., 1994b; Hou et al., 1995]. Although mutations in the X region of the HBV DNA have been suggested to lead to suppression of HBV replication and protein expression in the cells [Preisler-Adams et al., 1993; Uchida et al., 1995; Fukuda et al., 1996; Loeb et al., 1996; Moriyama, 1997], several factors involved in silent hepatitis B remain to be elucidated.

The first question that needs to be answered is: what is the actual level of HBV replication in patients with silent hepatitis B? The end-point dilution method [Shindo et al., 1991; Niitsuma et al., 1997] and the competitive PCR [Hagiwara et al., 1993] for the evaluation of viremia in patients with HBV or hepatitis C virus (HCV) infection has been used to determine viral concentrations in the serum and liver, but the range of viremia detection with these methods is limited. Furthermore, extremely low levels of HBV DNA cannot be detected accurately with these methods. In contrast, fluorometric quantitation of PCR products using a TaqMan chemistry PCR method [Heid et al., 1996], which enables the reporter fluorescent signal released from the hybridized probe to the target to be measured in real time, overcomes the difficulty of measuring such low HBV DNA concentrations. Therefore, it is now possible to determine the HBV DNA concentrations accurately in patients with silent hepatitis B using this novel fluorogenic PCR.

The second question is: what are the genetic charac-

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teristics of the HBV DNA in patients with silent hepatitis B? The HBV X region, which contains the core promoter, enhancer II, and two direct repeats is essential for HBV replication. An 8-nucleotide (nt) deletion of the HBV X region has been shown to be related to the pathogenesis of silent hepatitis B [Preisler-Adams et al., 1993; Uchida et al., 1995; Fukuda et al., 1996]. This deletion may suppress viral replication and expression, resulting in reduced antigen production. However, whether this deletion is common to all patients with silent hepatitis B is unclear. It is important to analyze the genetic changes of the HBV X region in silent hepatitis B.

Finally, the clinical characteristics of silent hepatitis B, such as the liver enzyme level changes, histology, immune responses to the virus, and route of infection, need to be clarified.

In this study, we analyzed two patients with chronic silent hepatitis B and studied their serum HBV DNA levels, genetic changes of the HBV X region, and clinical characteristics. These results provide new information about silent hepatitis B.

PATIENTS AND METHODS

Patients

Two patients who had been diagnosed previously as having non-B, non-C chronic hepatitis were investigated. Both patients were negative for any serologic markers indicating persistent infection with hepatitis viruses and had no history of blood transfusion, alcohol consumption, or drug abuse.

Patient 1 was a 27-year-old woman with a 7-year history of abnormal liver function tests. She was admitted to the Yamagata University Hospital in May 1991 complaining of general fatigue. Her serum alanine aminotransferase (ALT) level was 80 IU/l (normal < 30 IU/l). Tests for anti-hepatitis A virus (HAV) IgM (radioimmunoassay [RIA], Dainabot Co. Ltd., Tokyo, Japan), HBsAg (enzyme-linked immunosorbent assay [ELISA], Behring Inc., Marburg, Germany), anti-HBs (ELISA, Behring Inc.), anti-HBc (ELISA, Behring Inc.), hepatitis B e antigen (HBeAg) (RIA, Dainabot Co. Ltd.), anti-HBe (RIA, Dainabot Co. Ltd.), anti-HCV (The third-generation ELISA, Ortho Clinical Diagnostics Inc., Raritan, NJ), anti-hepatitis delta virus (HDV) (RIA, Dainabot Co. Ltd.), anti-cytomegalovirus IgM (ELISA, Behring Inc.), anti-herpes simplex virus IgM (ELISA, Behring Inc.), and anti-Epstein Barr virus IgM (ELISA, SRL Inc., Tokyo, Japan) were all negative. The negative anti-HBc assay was confirmed by RIA tests (Dainabot Co. Ltd.). The nested, reverse transcription (RT)-PCR using specific primers encoding the 5′ noncoding region (5′NC) of HCV [Widell et al., 1991] did not detect serum HCV RNA. Hepatitis G virus (HGV) RNA and TT virus (TTV) DNA were examined. The serum HGV RNA was not detected by a nested RT-PCR using specific primers encoding the 5′NC of HGV as described previously [Zhang et al., 1997]. The serum TTV DNA was not detected by a semi-nested PCR using primers derived from the conserved regions, as reported previously [Okamoto et al., 1998]. Antinuclear antibody, anti-smooth muscle antibody, and anti-mitochondrial antibody were all negative and the serum gamma-globulin level was within the normal range.

Patient 2 was a 30-year-old man who had complained of general fatigue for 1 month. His serum ALT level, determined at the outpatient clinic, was also high (557 IU/l) and he was admitted to the same hospital as patient 1 in January 1992. His serum ALT level on admission was 357 IU/l. He had received a vaccination of HBsAg and his serum anti-HBs was positive. He was negative for anti-HBs before vaccination. Tests for anti-HAV IgM, HBsAg, anti-HBc, HBeAg, anti-HBe, anti-HCV, HCV RNA, anti-HDV, HGV RNA, TTV DNA, anti-cytomegalovirus IgM, anti-herpes simplex virus IgM, and anti-Epstein Barr virus IgM were all negative. Anti-nuclear antibody, anti-smooth muscle antibody, and anti-mitochondrial antibody were all negative and the serum gamma-globulin level was within the normal range.

Immunohistochemistry

An indirect immunoperoxidase staining technique was used to detect HBsAg and HBcAg in the liver biopsy specimens, as described previously [Saito et al., 1992]. The primary antibodies against HBsAg and HBcAg used were purchased from Dako Co. Ltd. (Kyoto, Japan).

TaqMan Chemistry PCR for Quantitative HBV DNA Analysis

The TaqMan chemistry PCR procedure for quantitation of HBV DNA was developed at SRL Inc. (Tokyo, Japan) and this assay can detect as few as 2.2×10^2 HBV DNA copies/ml in real time. HBV DNA was extracted from 100 μl serum using the EX-R&D nucleic acid extraction kit according to the manufacturer's instructions (Sumitomo Metal Inc., Tokyo, Japan), redissolved in 10 μl water and heated at 60°C for 10 min before subjecting it to the PCR procedure. The amplification targets of the HBV DNA were the S and X regions, 174 nucleotides (nt) and 331 nt, respectively. The primers used for PCR amplification were 5′CACATCAGGATTCCTAGGACC3′ and 5′GGTGAGTGATTGGAGGTTG3′ for the S region and 5′ACGTCCTTTGTTTACGTCCCGT 3′ and 5′CCCAACTCCTCCCAGTCCTTAA3′ for the X region. The nonextendible fluorogenic hybridization probes (TaqMan probes) 5′CAGAGTCTAGACTCGTGGTGGACTTC3′ and 5′TGTCAACGACCGACCTTGAGGCATA3′ were designed for the S and X regions, respectively. A 45-μl aliquot of TaqMan PCR mixture (5 μl 10× PCR buffer; 1 μl each 10 mM dATP, 10 mM dGTP, and 10 mM dCTP; 1.25 μl 20 mM dUTP; 0.66 μl each 15 μM sense and anti-sense primers; 5 μl 3 μM TaqMan probe; 0.5 μl AmpliTaq Gold; 0.5 μl AmpErase uracil *N*-glycosylase [UNG]; 5 μl 25 mM $Mn(Oac)_2$ solution; 23.43 μl water) was mixed with 5 μl DNA solution in the well of PCR plate. The PCR conditions were as

follows: 50°C for 2 min for the contamination control with AmpErase UNG, 95°C for 12 min to deactivate the UNG, followed by 40 cycles of 95°C for 20 sec for denaturation and 60°C for 1 min for annealing and extension. The HBV DNA concentration was calculated compared with that of the standard DNA in real time by measuring the reporter dye fluorescent emission spectra cleaved from the fluorescent hybridization TaqMan probe using an ABI Prism 7700 Sequence Detector (Perkin Elmer Japan Applied Biosystems, Chiba, Japan).

Direct Sequencing of the HBV X Region

The serum samples in which HBV DNA was detected by TaqMan PCR were selected. A 100-μl aliquot of each patient's serum was mixed with 300 μl of lysis buffer (consisting of 12.0 mM Tris HCl buffer pH 8.0, 10.0 mM ethylenediamine tetraacetic acid [EDTA], 0.6% sodium dodecyl sulfate, and 120 μg/ml proteinase K) and incubated at 70°C for 3 hr. DNA was extracted with phenol/chloroform and was dissolved in 20 μl of water after ethanol precipitation. The entire HBV X region was amplified by a nested PCR using appropriate primer sets, as described previously [Uchida et al., 1995]. The amplified HBV DNA X region was purified using a QIAquick PCR purification kit (Qiagen Ltd., Hilden, Germany). Direct sequencing of the HBV DNA X region was performed by the dideoxy sequencing method using a Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, OH), as described previously [Uchida et al., 1995].

RESULTS

Hepatitis B Viremia and Serial ALT Levels

The clinical courses of the two patients, including their serial serum ALT levels and HBV viremia values are shown in Figure 1. The serum ALT level of patient 1 was persistently elevated and fluctuated during clinical follow-up over 7 years, except when she was pregnant. Liver biopsy was performed twice and both specimens showed chronic persistent hepatitis (CPH) with minimal necroinflammation without portal fibrosis. HBV DNA was amplified by TaqMan PCR for the S and X regions, in three and two samples, respectively, of the 15 tested. The mean HBV DNA concentrations (copies/ml) determined by S and X region analysis were 3.8×10^3 and 1.8×10^3, respectively (Fig. 1A). The serum ALT level of patient 2 fluctuated between the normal level of less than 30 IU/l and more than 300 IU/l during clinical follow-up over 2 years. A liver biopsy specimen showed CPH of the same appearance as that in patient 1. HBV DNA was amplified by TaqMan PCR for both the S and X regions in 2 of 17 samples tested and the mean HBV DNA levels (copies/ml) determined by S and X region analysis were 2.5×10^3 and 2.0×10^3, respectively (Fig. 1B).

Sequence Analysis of the HBV X Region

The entire X region was amplified successfully by the nested PCR using the patients' serum samples in which HBV DNA was detected by TaqMan PCR, followed by direct sequencing. Figure 2 shows the nt substitutions found in these patients. The consensus sequence had been determined in previous studies [Ono et al., 1983; Uchida et al., 1997]. In both patients, the start ATG codons of the X region (nt 1376) and precore region (nt 1816) were well preserved. The T-to-C mutation at the extreme 5′-terminus of the direct repeat (DR) 2 region was observed in patient 1. This mutation may be important, because DR2 has been implicated in the initiation of HBV DNA replication [Seeger et al., 1986] and, therefore, it may result in suppression of HBV DNA replication. This mutation was not found in our sequence study on the HBV X region in the sera of patients with HBsAg-positive chronic hepatitis B [Uchida et al., 1997] and it has been reported to be one of the unique point mutations observed in patients with silent hepatitis B [Uchida et al., 1995]. Patient 2 exhibited an X region with a 155-nt deletion, which included DR2 and the core-promoter sequence, coexisting with the full-length X region. The 8-nt deletion, which has been reported to be characteristic of silent HBV infection [Preisler-Adams et al., 1993; Uchida et al., 1995; Fukuda et al., 1996], was not detected in either patient.

Expression of HBV-Associated Antigens in the Liver

Diffuse, but faint HBsAg staining was observed within the cytoplasm of some hepatocytes of liver specimens of the two patients, but no HBcAg staining of their liver tissues was observed. Figure 3 shows the expression of HBsAg in the first liver biopsy specimen of patient 1.

Investigation of Vertical Transmission of Silent HBV

Patient 1 has two children, a 6-year-old boy and a 3-year-old girl, and their sera were examined for serologic HBV markers and their serum ALT levels were determined. Their ALT levels were within the normal range and tests for HBV markers, including HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc, were all negative. Then, their sera were analyzed by TaqMan PCR and the S gene of HBV DNA was amplified successfully by TaqMan PCR in one of two serial samples obtained from the girl. HBV DNA was not amplified in one sample obtained from the boy. The serum HBV DNA concentration of the girl was low, 4.4×10^3 copies/ml. Therefore, vertical transmission of silent HBV from the mother to her child had almost certainly occurred.

DISCUSSION

The two patients described above had been diagnosed as having non-B, non-C chronic hepatitis, as all tests for diagnostic viral markers in their sera were negative. However, TaqMan PCR revealed that they were actually infected with HBV, although their serum HBV DNA levels were as low as approximately 10^3 copies/ml. Although low-level HBV replication was

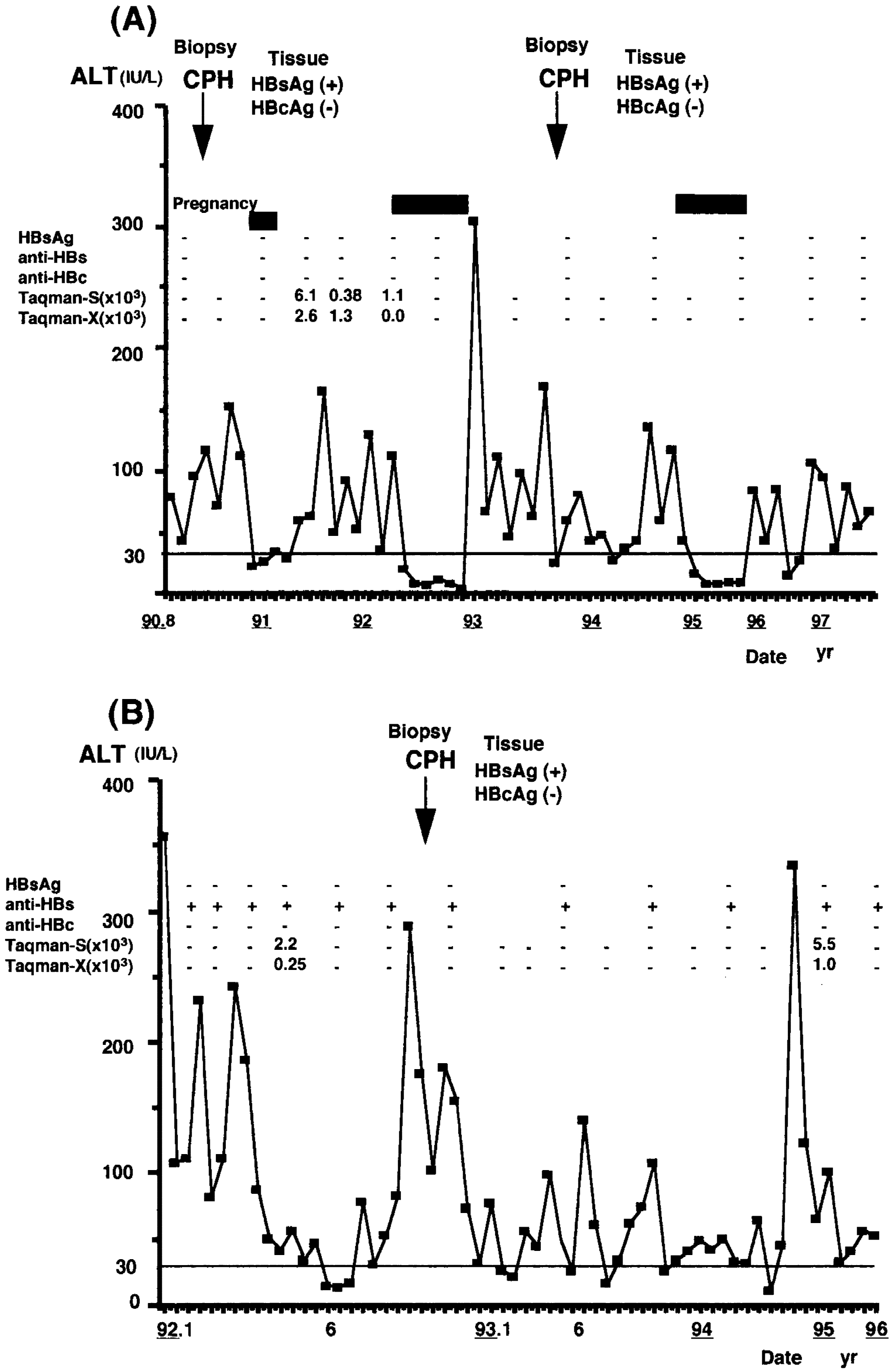

Fig. 1. Serial serum alanine aminotransferase (ALT) levels (normal < 30 IU/l), serum hepatitis B virus (HBV) markers, HBV DNA concentrations, and liver histology. The HBV marker and HBV DNA levels were determined by an enzyme-linked immunosorbent assay and TaqMan chemistry PCR, respectively. **(A)** The serum ALT levels of patient 1 fluctuated during follow-up over 7 years, except when she was pregnant. Liver biopsy was performed twice and revealed chronic persistent hepatitis (CPH) with minimal necroinflammation without fibrosis. Tests for HBV markers, including hepatitis B surface antigen (HBsAg), anti-HBs, and anti-hepatitis B core antibody (anti-HBc), were negative throughout the observation period, but HBV DNA was detected occasionally by TaqMan PCR for the S and X regions and their concentrations were approximately 10^3 copies/ml. **(B)** The serum ALT levels of patient 2 fluctuated and he was negative for both HBsAg and anti-HBc, indicating persistent HBV infection. This patient had developed anti-HBs after vaccination. HBV-DNA was detected by TaqMan PCR for both the S and X regions and the concentrations were low, approximately 10^3 copies/ml.

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            1380                1400                1420                1440                1460
        ATGGCTGCTCGGGTGTGCTGCCAACTGGATCCTGCGCGGGACGTCCTTTGTCTACGTCCCGTCGGCGCTGAATCCCGCGGACGACCCGTCTCGGGGCCGT
Case 1  ---------A----------------------------------T------------------------------------------A------------
Case 2  ---------A-----------------------A----------T------------------------------------------C--C--------C
Case 2D ---------A-----------------------CTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAA

            1480                1500                1520                1540                1560
        TTGGGCCTCTACCGTCCCTTGCTTTCTCTGCCGTTCCAGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCCGTCTGTGCCTTCTCATCTGCCGG
Case 1  -----A------------C-T--CCG-------------------------------------------------------------------------A
Case 2  -----G--------C--GC-T--CCG--------A--GA-----------A-------------------A-----------------------------
Case 2D ACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCC/////////////////////////////////////////////////////////////////

            1580         #      1600                1620                1640                1660
        ACCGTGTGCACTTCGCTTCACCTCTGCACGTCGCATGGAGACCACCGTGAACGGCCACCAGGTCTTGCCCAAGCTCTTACATAAGAGGACTCTTGGACTC
Case 1  G---------------C------------------------------------C-------------------G--------------------------
Case 2  ----------------------------T----------A-------------C-----G-AA-C--------G----G--------------------T
Case 2D //////////////////////////////////////////////////////////////////////////////////////////----------

            1680                1700                1720                1740                1760
        TCAGCAATGTCAACAACCGACCTTGAGGCATACTTCAAAGACTGTTTGTTTAAAGACTGGGAGGAGTTGGGGGAGGAGATTAGGTTAAAGGTCTTTGTAC
Case 1  --------------G------------------------------G------------------------------------------------------
Case 2  ----------C---G------------------------------G-------T--G---------C---------------------------------
Case 2D --------------G--------------G-----------------------G----------------------------------------------

            1780                1800                1820
        TAGGAGGCTGTAGGCATAAATTGGTCTGTTCACCAGCACCATGCAACTTTTTCACCTCTGCCTAA
Case 1  -----------------------------------------------------------------
Case 2  -----------------------------------------------------------------
Case 2D -----------------------------------------------------------------
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Fig. 2. Nucleotide sequence of the entire hepatitis B virus (HBV) X region. The top line indicates the consensus nucleotide sequence and the lower nucleotides are the substitutions found in our two patients. Numbering starts from the EcoRI site. A T-to-C mutation (#) located at the extreme 5′-terminus of direct repeat 2 (DR2) (TTCACCTCTGC to CTCACCTCTGC) was observed in patient 1. Patient 2 had two species of HBV DNA, one of wild-type size and an X deletion mutant (D) showing a 155-nt deletion (/), which included DR2 and core-promoter sequence.

speculated to occur in patients with silent hepatitis B [Uchida et al., 1995], in this study, we quantitated, for the first time, the serum HBV DNA levels in such patients. HBV infection was also confirmed by the demonstration of HBsAg expression in the liver.

The recent development of PCR technology has facilitated the evaluation of HBV viremia at the molecular level. TaqMan chemistry PCR, which provides fluorometric quantitation of PCR products in real time, enables us to perform accurate and wide-ranging quantitation with a low risk of contamination, which often accompanies the PCR procedure [Heid et al., 1996]. HBV viremia was not detected constantly, even though this sensitive PCR was used for these two patients, but their viremia levels, 10^3 copies/ ml, could have not been detected accurately by conventional PCR quantitation.

Specific HBV mutants have been shown to be a causative agents of silent hepatitis B [Preisler-Adams et al., 1993; Kremsdorf et al., 1993; Feitelson et al., 1994; Uchida et al., 1994b; Fukuda et al., 1996]. Complete nucleotide sequence analyses of such mutants revealed a characteristic 8-nt deletion in the HBV X region [Uchida et al., 1995]. This 8-nt deletion truncates the X protein from 154 to 134 amino acids (aa) and the truncated X protein seems to diminish the original transactivating function of the X protein [Repp et al., 1992; Uchida et al., 1997]. Intact X protein transactivates the promoters and enhancers of HBV DNA, promoting its replication and expression [Seeger et al., 1986]. This deleted 8-nt sequence also constitutes part of the enhancer II/core promoter located at nt 1591–1851 and nt 1687–1805, respectively [Preisler-Adams et al., 1993; Uchida et al., 1995; Fukuda et al., 1996]. Thus, the deletion within HBV X region may result in suppression of viral replication and expression, eventually leading to negative serum HBV-related antigen and antibody test results. This hypothesis has been confirmed by an in vitro transfection study, in which transfection of several plasmids containing mutant HBV with an 8-nt (position 1763–1770) or a 20-nt (position 1753–1772) deletion into HepG2 cells reduced the HBsAg, HBcAg, and HBeAg levels of cultured cells [Moriyama, 1997].

Sequence analysis of the HBV DNA from our two patients did not show the 8-nt deletion, characteristic of the HBV DNA reported previously in patients with silent hepatitis B [Uchida et al., 1995]. This discrepancy may be due to the sequencing procedure we used that detected the major population of the virus and because the PCR may not have amplified a coinfected and smaller amount of an HBV mutant with an 8-nt deletion in its X region. Our sequencing data showed several interesting mutations of the X region. In patient 1, DR2 showed a T-to-C mutation at its extreme 5′-terminus. This point mutation was found in a patient with chronic silent hepatitis B [Uchida et al.,

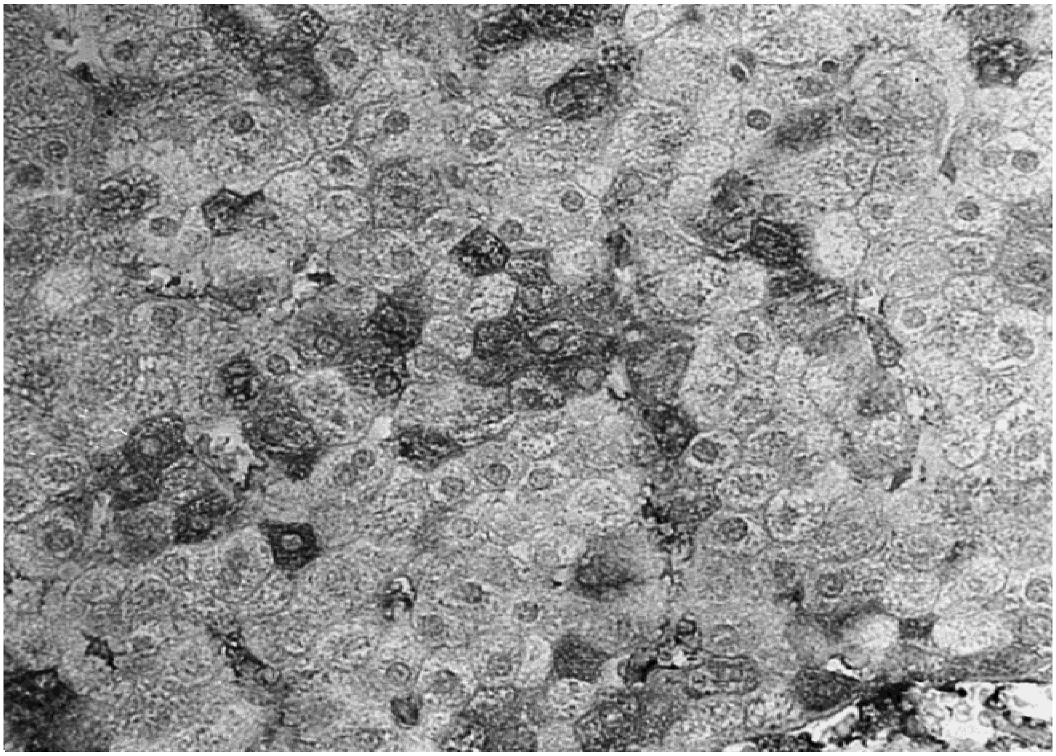

Fig. 3. Expression of hepatitis B surface antigen (HBsAg), detected by immunoperoxidase staining, in the first liver biopsy specimen of patient 1. Diffuse, but faint, HBsAg immunostaining was detected in the cytoplasm of the hepatocytes (×320).

1995]. Furthermore, this point mutation was not detected in our previous sequence study on the X region in patients with HBsAg-positive hepatitis B [Uchida et al., 1997]. The 5′-terminus of DR2 is the initiation point of HBV DNA synthesis [Seeger et al., 1986], so this point mutation would be expected to reduce HBV replication significantly. In patient 2, a coexisting much larger X region deletion that included the DR2/core promoter associated with wild-type HBV DNA was detected. This patient had an anti-HBs as a result of vaccination and this antibody seemed ineffective for neutralizing the HBV mutant. A previous study showed that anti-HBs generated by vaccination did not prevent infection by an HBV mutant in β-thalassemia patients, in whom an HBV mutant with a long deletion of the X region was found to be involved in the pathogenesis of serologically negative hepatitis B [Feitelson et al., 1994]. Mutant HBV DNA with an X-region deletion, which may be nonfunctional in some aspects of replication and infection, probably transrepresses wild-type HBV DNA, because both these HBV DNAs in the sera of patients with chronic silent hepatitis B are occasionally PCR amplified (unpublished data).

The expression of HBsAg was confirmed by immunoperoxidase staining of liver specimens, but HBcAg was not detected in the liver of either patient. As immunohistochemical detection of HBcAg correlated well with viral replication [Chu and Liaw, 1987; Uchida et al., 1994a], such negative HBcAg staining corresponds to low-level HBV replication in these two patients. Low-level replication and expression of HBV DNA were probably responsible for the mild hepatic necroinflammation in these two patients, because the target antigen of chronic HBV infection is considered to be HBcAg [Chu and Liaw, 1987; Saito et al., 1992], which was expressed minimally in their livers. We also analyzed the HBV DNA viremia level using TaqMan PCR in three patients with both HBsAg- and HBeAg-positive chronic hepatitis B and their mean viral concentration was higher than 10^9 copies/ml (unpublished data). These findings suggest that HBV replication is suppressed considerably in the liver of patient with silent hepatitis B.

Recently, HGV [Linnen et al., 1996] /GB virus C (GBV-C) [Simons et al., 1995] and TTV [Nishizawa et al., 1997] were discovered as candidates for non-A to E hepatitis viruses. It has been shown that most HGV/GBV-C infections are not associated with hepatitis [Alter et al., 1997]. The etiological importance of TTV in association with liver diseases has not been characterized [Cossart, 1998]. We investigated the superinfection of these viruses in these two patients, but neither HGV RNA nor TTV DNA was detected in the serum by PCR.

In conclusion, we demonstrated low-level HBV replication in two patients with chronic silent hepatitis B by quantitating their serum HBV DNA levels. Despite the low serum HBV DNA levels, vertical transmission of silent HBV had almost certainly occurred. Further studies are needed to elucidate the immunological

mechanisms of liver injury that correlate with persistent infection in silent hepatitis B.

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